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OPPT-2002-0029-0023

Amphibian Metamorphosis Assay Detailed Review Paper

Endocrine Disruptor Methods Validation Subcommittee
July 2002

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EPA

Detailed Review Paper: AMPHIBIAN METAMORPHOSIS ASSAY FOR ENDOCRINE DISRUPTION

WORK PERFORMED BY:

Battelle
Putting Technology To Work

Fort Environmental Laboratories, Inc.

On behalf of the United States Environmental Protection Agency

EPA CONTRACT NUMBER 68-W-01-023

METHODS USED IN THIS ANALYSIS

- On-line Literature Search (December 2001)
  - Encompassed searching of traditional literature databases, contacting specific experts in the field, and evaluating other presonal communication
  - Databases included, Medline/PubMed, biological Abstracts, Chemical Abstracts, and Toxline using key word and author search strategies
  - General search was performed initially using the key phrases amphibian metamorphosis and amphibian thyroid
  - Search was refined to Key Words - endocrine disruptor, thyroid impairment, TH analysis, cDNA techniques, and culture methods using Boolean operators "and" and "or"
  - Approximately 10000 records were refined down to 1000 papers that were reviewed

METHODS USED IN THIS ANALYSIS Cont.

Telephone/Email Consultations:

- Drs. Tyrone Hayes, James Burkhart, Robert Denver, and Robert Granger
  - Yielded 9 new documents not originally included in initial literature search
  - Each were included in various sections of the DRP

METHODS USED IN THIS ANALYSIS Cont.

Interviews With The Following Experts:

Dr. Robert Denver
Dr. Brent Palmer
Dr. Joe Bidwell
Dr. Jim Burkhart

METHODS USED IN THIS ANALYSIS Cont.

External/Internal Peer Review

- Dr. Joe Bidwell - Oklahoma State Univ.
- Dr. Brent Palmer - U of Kentucky
- Mr. Michael Blanton - Battelle
- EPA Technical Experts
  (principally Dr. Joe Tietge, MED)

OVERVIEW AND SCIENTIFIC BASIS OF AMPHIBIAN METAMORPHOSOSIS ASSAY (ENDOCRINE CONTROL OF THYROID AXIS)

- Amphibian metamorphosis is well understood and generally involves morphological, biochemical, and molecular changes
- These changes result in resorption, remodeling, and creation of new tissues
- Thyroid axis control of metamorphosis in amphibians involves the CNS, hypothalamus, pituitary gland, thyroid gland, TH transport proteins, TRs, and transcriptional elements
- Many aspects of the thyroid axis are conserved amongst chordates at both the morphological and molecular levels enhancing the use of an amphibian as a general vertebrate model for evaluating thyroid disruption
- The Amphibian Metamorphosis Assay will be useful in evaluating thyroid perturbation.

Overview of Metamorphic Periods in Anurans

- Premetamorphosis
  - Characterized as phase of embryogenesis and early tadpole growth
  - Thyroid gland is developing
- Prometamorphosis
  - Larvae acquires TH synthesis capacity and is characterized by concentration of endogenous TH
- Metamorphic Climax
  - Characterized by peak levels of endogenous TH and rapid and drastic morphological changes occur (i.e., tail resorption)

Test Species

- Anurans
  - Pipids
    - South African clawed frog (*Xenopus sp.*)
      - *X. laevis*
      - *X. tropicalis*
  - Ranids
    - Leopard frog (*Rana pipiens*)
  - Hyperoliids
    - *Hyperolius sp.*
- Urodeles

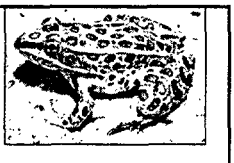
Xenopus laevis

- Native to Africa, south of Sahara desert
- Extensively used in scientific research and can be obtained from US vendors
- Adult males are ca. 10 cm and females 15 cm in length (metamorphic tadpoles are 2-3 cm in length)
- Can be kept in breeding condition all year and are bred in the lab using hCG
- Females produce 1500 embryos per breeding
- May be re-bred every two months and are productive for 3 to 5 years
- Purely aquatic species

Xenopus tropicalis

- Native to southern tip of Africa
- Similar to X. laevis with the following exceptions:
  - Physically smaller
  - Develop more rapidly (can be cultured at higher temperature)
  - Diploid genome
  - Somewhat greater embryo production
  - Reproductively mature in 4-5 months (X. laevis requires 1-2 years)

Rana pipiens



- Native to North America
- Breeding season ranges from March to April
- Aquatic and terrestrial life phase - more difficult to raise and breed in lab
- Sexual maturity in 1 to 2 years
- Produces Eggs per egg masses of 1500 to 4000 in the wild

Hyperoliid sp.

- Native to Africa, known as the reed frog
- Undergoes ontogenic color change as the result of secondary sexual development
- No commercial source of species
- Limited culturing information available

Urodeles

- Includes "non-frog" species, such as salamanders, newts, and axolotls
- Native to North America and other locations across the globe.
- Breeding occurs in the Early Spring months depending on temperature
- Produce 100-200 relatively large embryos per breeding
- Limited culturing information available

Xenopus laevis

Strengths

- Large database on all aspects of development, reproduction, metamorphosis, including information at molecular level
- Ease of culture
- Breeds in laboratory repeatedly with hormonal stimulus
- High productivity
- Mapped genome
- Many laboratories are familiar with culture and testing

Weaknesses

- Relatively long life cycle
- Oligotetraploid genome

Xenopus tropicalis (compared to *X. laevis* only)

Strengths
- Relatively short life cycle
- Rapid development
- Diploid genome
- Good transgenic capacity
- Greater breeding output

Weaknesses
- More challenging animal husbandry
- Less scientific information availablecs
- Disease susceptibility?
- Availability

Rana pipiens

Strengths
- Native species
- Reasonable database
- Relatively short metamorphic period for native species
- Terrestrial and aquatic life phases

Weaknesses
- More difficult animal husbandry and breeding
- Limited testing experience

Urodeles

Strengths
- Represent different Order
- Native species
- Terrestrial and aquatic life phases

Weaknesses
- More difficult animal husbandry and breeding
- Limited testing experience
- Limited database

Hyperolius sp.

Strengths

- External endpoints
- Seemingly straight-forward endpoints
- Suitable animal husbandry
- Connection to sexual development

Weaknesses

- Availablity
- Limited testing experience
- Limited database
- Does not directly measure thyroid dysfunction

Routes of Administration of Chemical Exposure

- Aqueous
- Dietary exposures
- Parenteral
  - -intravenous
  - -intramuscular

Potential Exposure Periods

- Exposure from premetamorphosis through metamorphic climax
  - 35+ days
- Exposure from prometamorphosis through metamorphic climax
  - 28+ days
- Exposure during prometamorphosis
  - ca. 14 days
- Exposure during metamorphic climax
  - ca. 14+ days

Exposure Period

- Premetamorphosis
  - No thyroidal activity
  - This phase only considers development of the thyroid gland
- Prometamorphosis
  - Potentially the most sensitive stage as the organism is acquiring thyroid activity
  - Hind limb development occurs
- Metamorphic climax
  - Substantial morphological changes occur
  - TH saturation
    - May be difficult to distinguish effects due to TH saturation
    - Reduced sensitivity anticipated

Measurement Endpoints Considered

- Morphological and Histological
  - Rate of Development (developmental delay)
    - Must discriminate between thyroidal and non-thyroidal responses
  - Hind Limb Development and Differentiation
  - Tail Resorption
  - Thyroid gland histopathology
    - Hyper- and hypotrophy, and hyper- and hypoplasia
- Biochemical
  - TH
  - TH precursors

Measurement Endpoints Considered (continued)

- Molecular
  - cDNA Techniques
    - Single Gene Expression
      - RPA
      - RT-PCR
    - Multiple Gene Expression
      - Differential Display
      - Gene Arrays
  - Transgenic Lines
  - Organ and Cell Culture
  - Receptor binding Assays

MEASUREMENT OF BIOCHEMICAL ENDPOINTS

- TH and TH precursors
  - Radioimmunoassay (RIA)
  - Enzyme-linked Immunosorbent Assay (ELISA)
  - Liquid/Gas Chromatography with Mass Selective Detection (LC/GC-MS)

Relevant Gene Expression Techniques

- Single Gene Expression
  - RT-PCR
  - Genes Considered: TRbeta, ST3, transthyretin
- Multiple Gene Expression
  - Gene Arrays
  - Gene Familes Considered: TR familes with TRE, ST3

Anticipated Endpoint Sensitivity

- Molecular/Biochemical (most sensitive)
- Histological
- Morphological (least sensitive)

Species Selection Criteria

- Species must be amenable to continuous culture in the lab
- Reproduction must be continuous throughout the year or be inducible through hormonal treatments
- Larvae must be able to be routinely reared to predetermined developmental stages
- Relatively fast rate of development
- The endpoints that will be used must be supported by a sufficient database clearly indicating their relevance

Additional Beneficial Criteria

- Knowledge of genetic information
- Biochemical information known about the endocrine axis (HPT in this case)
- Metabolism information (TH homeostasis)

Recommended Test Species

- The only species that meets the minimal criteria established is *X. laevis.*

CANDIDATE PROTOCOLS

1) Xenopus 28-day full metamorphosis assay (proposed by OECD, 2001)
   1) Note - theoretically this assay should require 50+ days
2) 14-day metamorphic climax assay (as described in Fort et al., 2000)
3) 14-day prometamorphosis assay (as described by Tietge et al., personal communication)

14-Day Prometamorphosis Assay

- Chemical Exposure - 14 Days through end of premetamorphosis and onset of prometamorphosis
- NF Stage 51 to 54
- Exposure Measurement Endpoints
  - Thyroid gland histopathology, hind limb emergence (rate and normalcy), TH and TH precursor levels, and differential gene expression via gene array from tissue punch samples (i.e., TR beta family)
- Interpretation of Results

Significant Data Gaps

1. Responses, at the organismal and suborganismal levels, to known thyroid agonists and antagonists
2. Which endpoints provide most meaningful information regarding effects due to thyroid-based mechanisms
3. The time course of responses
4. Sensitivity of the measurement endpoints
5. Relationship between quantitative changes in molecular activity and observed changes in thyroid homeostasis
6. Dynamic range of thyroid axis homeostasis and its relationship to gross morphological, histological, molecular, and biochemical changes

IMPLEMENTATION CONSIDERATIONS

- Animal Welfare Considerations
- Test Facility Capablities
- Prevalidation Studies (in general accordance with ICCVAM guidelines)
  - Phase I - Final definition and development of the recommended endpoints
    - Most work will be required with molecular techniques including construction of the gene arrays
  - Phase II - Preliminary protocol development for exposure and measurement endpoints.

IMPLEMENTATION CONSIDERATIONS (continued)

- Prevalidation Studies
  - Phase II (cont.) - Evaluate a set of three known thyroid agonists and antagonists using preliminary protocols
  - Phase III - Evaluate data, review and revise preliminary protocols as necessary
  - Phase IV - Test and additional set of three test substances with anecdotal or unknown information regarding thyroid axis activity
  - Phase V - Review data, revise protocol accordingly to generate Final Protocol for use in interlaboratory GLP validation studies

Questions

1) Does the EDMVS agree that the Prometamorphosis Assay with Xenopus is the appropriate amphibian assay to recommend for further implementation?

Questions (continued)

2) Does the EDMVS agree that pre-validation efforts should be phased as described in the DRP and targeted to address the following:

a. establish organismal and sub-organismal responses to established thyroid agonists and antagonists,
b. determine which endpoints are diagnostic of thyroid-based mechanisms,
c. ascertain the general time course of responses,
d. establish the sensitivity of the measurement endpoints, and
e. establish the dynamic range of thyroid axis related responses?

Questions (continued)

3) Does the EDMVS agree with using thyroxine as a thyroid agonist and perchlorate, propylthiouracil, and amiodarone as thyroid antagonists for evaluating the performance of this assay?

4) Does the EDMVS have suggestions to improve the DRP?